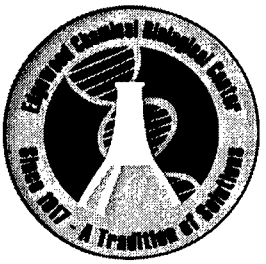

EDGEWOOD

CHEMICAL BIOLOGICAL CENTER

U.S. ARMY RESEARCH, DEVELOPMENT AND ENGINEERING COMMAND

ECBC-TR-458

STANDARDIZATION OF THE 3-(4,5-DIMETHYLTHIAZOL-2-yl)-5-(3-CARBOXYMETHOXYPHENYL)-2-(4-SULFOPHENYL)-2H-TETRAZOLIUM, INNER SALT (MTS) ASSAY FOR THE SK-N-SH, KYSE-30, MCF-7, AND HeLa CELL LINES

Jennifer G. Catalano
James J. Valdes
Darrel Menking

RESEARCH AND TECHNOLOGY DIRECTORATE

Kyle Hubbard
Averell L. Gnatt

UNIVERSITY OF MARYLAND AT BALTIMORE
Baltimore, MD 21201

July 2006

ABERDEEN PROVING GROUND, MD 21010-5424

DISCLAIMER

The findings in this report are not to be construed as an official Department of the Army position unless so designated by other authorizing documents.

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	2. REPORT TYPE	3. DATES COVERED *(From - To)*
XX-07-2006	Final	May 2001-May 2003

4. TITLE AND SUBTITLE
Standardization of the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, Inner Salt (MTS) Assay for the SK-N-SH, KYSE-30, MCF-7, and HeLa Cell Lines

5a. CONTRACT NUMBER

5b. GRANT NUMBER

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Catalano, Jennifer G.; Valdes, James J.; Menking, Darrel (ECBC); Hubbard, Kyle; and Gnatt, Averell L. (University of Maryland at Baltimore)

5d. PROJECT NUMBER
NONE

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) AND ADDRESS(ES)
DIR, ECBC, ATTN: AMSRD-ECB-RT-BM, APG, MD 21010-5424
University of Maryland at Baltimore, Baltimore, MD 21201

8. PERFORMING ORGANIZATION REPORT NUMBER
ECBC-TR-458

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution is unlimited.

13. SUPPLEMENTARY NOTES

14. ABSTRACT
One common way to examine toxicity *in vitro* is to measure the effect on cell viability. In one such assay, the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt MTS reagent is bioreduced to a formazan product in living cells. Various cell lines may have differing abilities to reduce MTS; therefore, standardization should be carried out for each one. To optimize the assay, the toxicity of MTS, the linear range for signal versus cell number, and a method of background noise reduction were determined. The MTS reagent decreased cell number after 25 hr. The linear range for the neuronal SK-N-SH, esophageal KYSE-30, breast MCF-7, and cervical HeLa cell lines were established. Finally, media containing no phenol red significantly reduced background noise compared to media with phenol red.

15. SUBJECT TERMS
Toxicogenomics
Toxicology
Toxic Industrial Chemicals
Cell Cultures

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			Sandra J. Johnson
U	U	U	UL	15	19b. TELEPHONE NUMBER *(include area code)* (410) 436-2914

Blank

PREFACE

The work described in this report was authorized under Career Related Experience in Science and Technology (CREST) in support of the Joint Program Office for Biological Defense. The work was started May 2001 and completed May 2003.

The use of either trade or manufacturers' names in this report does not constitute an official endorsement of any commercial products. This report may not be cited for purposes of advertisement.

The report has been approved for public release. Registered users should request additional copies from the Defense Technical Information Center; unregistered users should direct such requests to the National Technical Information Service.

Blank

CONTENTS

FIGURES

TABLES

STANDARDIZATION OF THE 3-(4,5-DIMETHYLTHIAZOL-2-yl)-5-(3-CARBOXYMETHOXYPHENYL)-2-(4-SULFOPHENYL)-2H-TETRAZOLIUM, INNER SALT (MTS) ASSAY FOR THE SK-N-SH, KYSE-30, MCF-7, AND HeLa CELL LINES

1. INTRODUCTION

The MTT [3-(4 dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] is a reagent that has been used to evaluate cell viability and cytotoxicity.[1] In living cells, MTT is converted to a colored formazan product which can be measured via spectroscopy. A bio-reduction reaction has been shown to take place in mitochondria by succinate dehydrogenase,[2] and other reports have shown that this reaction also occurs extra-mitochondrially.[3] The principle of the assay is that the more viable the cells, the more they will be able to convert the MTT substrate to product, resulting in a higher absorbance output signal.

Recently, the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) reagent, which is structurally similar to MTT, was introduced. The principle of the MTS assay is the same, and it is also converted to a colored formazan product.[4] However, there are two main advantages of using MTS over MTT. First, the MTS converted product is aqueous soluble, whereas MTT's product requires an organic solvent. Using the MTS reagent involves fewer steps and therefore potentially decreases the variability in the experiment. Second, once a harsh organic solvent is added to solubilize the formazan product of MTT, the experiment is terminated. In the case of MTS, once it has been added to cells, the plate can be read and placed back in the incubator for further color development. In the event that the signal is not strong enough, more time in the incubator will result in more MTS reagent being converted to product and thus a stronger signal. For example, Zhai et al. have demonstrated that it is possible to take several measurements on the same culture of parasites.[5]

Cell viability assays are a common method of measuring general outcome when cells are exposed to a toxicant. In order to carry out the assay effectively, the amount of cells per 96-well plate must be determined. If there are too few cells per well, there will not be a large enough signal to differentiate signal from background noise. If there are too many cells, there are two potential problems. The first is that an unwanted variable of media depletion is added to the experiment. The cells quickly use up the vital nutrients in the media and their growth rate decreases. If the effect of a toxicant on cell growth is being examined, then one would be studying two effects, the effect of media depletion and the effect of the toxicant on cell growth, which is undesirable. The cells may be affected due to media depletion and not because of any toxicant effect. Media depletion obscures the true effect of the toxicant. The second problem with a large cell number is that the system becomes saturated. If output signal (absorbance values) versus cell number is plotted, there is some range in which the relationship is linear. If there is a very high cell number, all of the MTS substrate is converted to product, and the output signal becomes the same for different cell numbers. If the signal is the same for different cell numbers, one cannot determine whether or not a toxicant has caused cell numbers to decrease. For this reason, the optimal range of cell number should be determined. The objectives are threefold: determine (1) the linear range of the MTS assay for four cell lines; (2) whether the

MTS reagent itself is toxic to cells; and (3) if there is a method for decreasing background noise of the assay.

2. MATERIALS AND METHODS

2.1 Cell Lines.

Four cell lines were used: (1) SK-N-SH neuroblastoma cells (ATCC, Manassas, VA) were grown in media containing 10% fetal bovine serum (FBS), 1% penicillin-streptomycin (10,000 U/ml), 1% sodium pyruvate (10X 100 mM), 0.1% nonessential amino acids (100 X, 10 mM), and minimum essential medium with Earle's salts and L-glutamine; (2) the KYSE-30 esophageal squamous cell carcinoma line was established by Shimada *et. al.* (6) (kind gift Steve Meltzer, University of Maryland School of Medicine) and was grown in 10% FBS, 1% penicillin-streptomycin (10,000 U/ml), and a 1:1 ratio of F-12 Nutrient mix and RPMI 1640; (3) MCF-7 breast cells (ATCC, Manassas, VA) were grown in 10% FBS, 1% penicillin-streptomycin (10,000 U/ml), and Dulbecco's modified Eagle medium with high glucose, L-glutamine, and sodium pyruvate; and 4) HeLa cervical cells (ATCC, Manassas, VA) were grown in 10% FBS, 1% penicillin-streptomycin (10,000 U/ml), and Dulbecco's modified Eagle medium with high glucose, L-glutamine, and sodium pyruvate. All reagents were purchased from Invitrogen Corp. (Carlsbad, CA). All cells were grown in 5% CO_2 at 37 °C.

2.2 Standard Curves.

Cells were plated in two 96 well plates, then 24 hr later, cells in plate 1 were detached from their surface using 0.25% trypsin-EDTA solution. Four replicate wells for each cell number plated were counted via a hemacytometer. For cells in plate 2, 100 µL of new media plus 20 µL MTS (Promega Corp., Madison, WI) was added. After 1-, 2-, 3-, and 4-hr later, the plates were measured on an ELx 808 microplate reader (Bio-Tek Instruments, Winooski, VT). Absorbance at 490 nm ± SEM versus cell number is reported.

2.3 Effect of MTS on Cell Number.

HeLa cells were plated in different numbers into two 96 well plates with 100 µL media/well. After 24 hr, 20 µL MTS was added to cells in one plate but not the other. After 25 hr, cells were counted via a Coulter Counter (Beckman Coulter, Inc., Miami, FL). A two way Analysis of Variance (ANOVA) was carried out using Prism software (GraphPad Software Inc., San Diego, CA).

2.4 Background Signal Comparison.

One hundred microliters of media with phenol red (10% fetal bovine serum, 1% penicillin-streptomycin (10,000 U/mL), 1% sodium pyruvate (10 x 100 mM), 0.1% nonessential amino acids (100 x 10 mM), and minimum essential medium with Earle's salts

and L-glutamine) was placed in four wells of a 96 well plate. One hundred microliters of media without phenol red (minimum essential medium with Earle's salts) was placed into four adjacent wells in the same 96 well plate. Twenty microliters of MTS was added to each of the wells and incubated (1 hr at 37 °C). The plate was then measured at 490 nm via an ELx 808 microplate reader (Bio-Tek instruments, Winooski, VT). Data analysis was carried out using the Student's unpaired t test ($\alpha = 0.05$).

3. RESULTS

3.1 Standard Curves.

The data indicate a relationship between absorbance signal and cell number, and there is a range in which this relationship is linear. The standard curves for the following are shown below: SK-N-SH cells (Figure 1), 40-449 cells (Figure 2), MCF-7 cells (Figure 3) and HeLa cells (Figure 4).

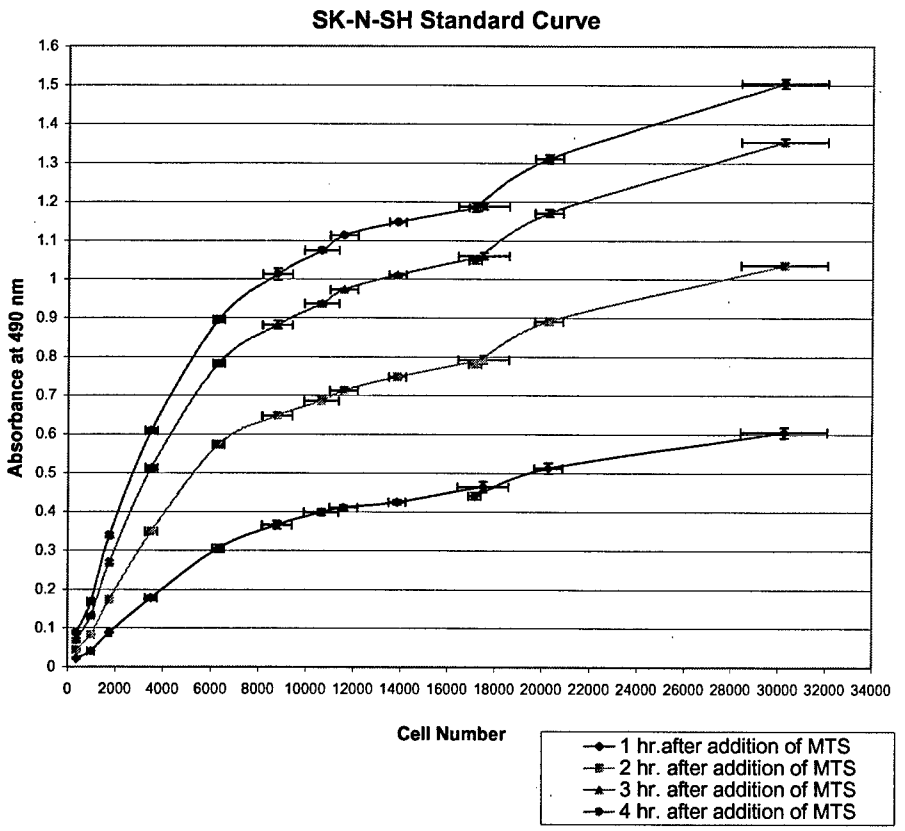

Figure 1. SK-N-SH Cell Standard Curve. Mean absorbance quadruplicate values at 490 nm ± SEM are shown at 1, 2, 3, and 4 hr after MTS addition.

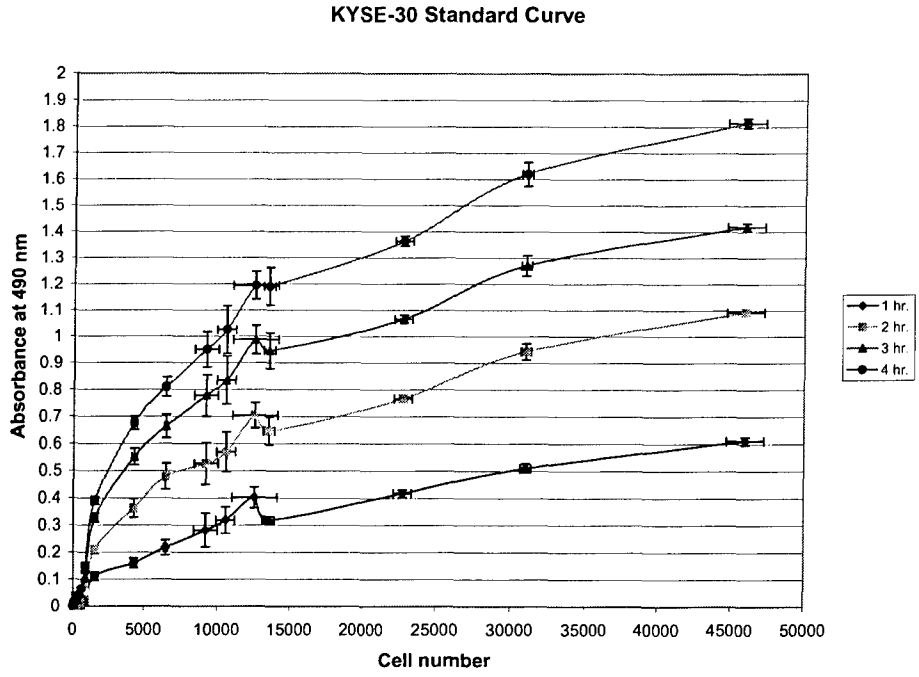

Figure 2. KYSE-30 Cell Standard Curve. Mean absorbance quadruplicate values at 490 nm ± SEM are shown at 1, 2, 3, and 4 hr after MTS addition.

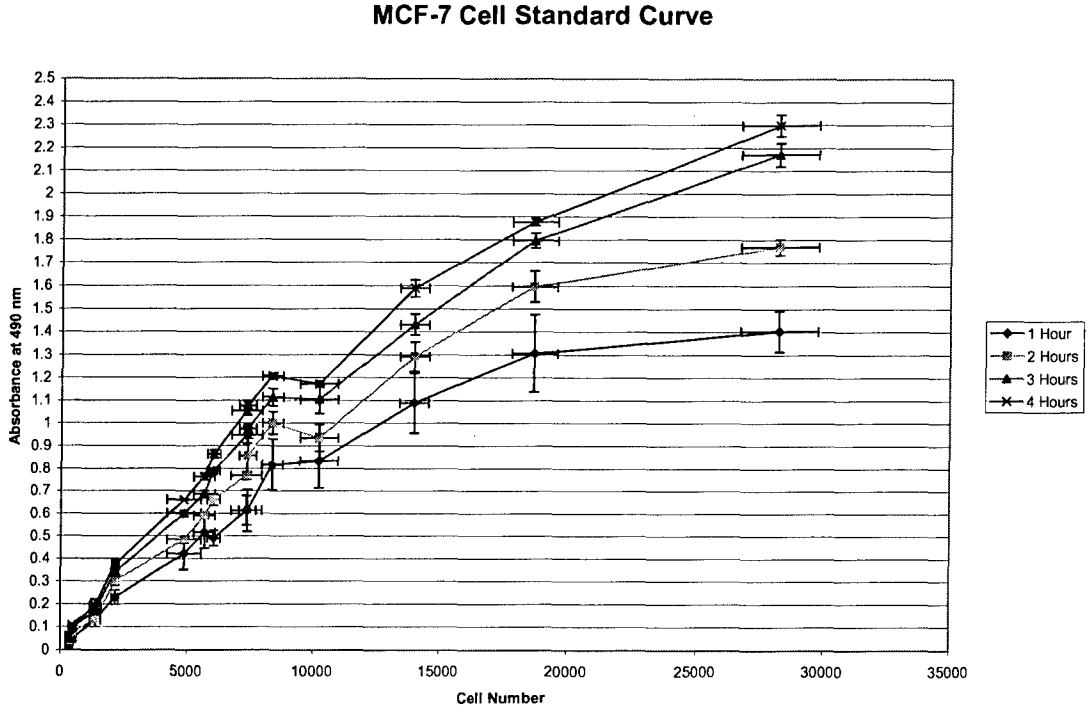

Figure 3. MCF-7 Cell Standard Curve. Mean of quadruplicate absorbance values at 490 nm ± SEM are shown at 1, 2, 3, and 4 hr after MTS addition.

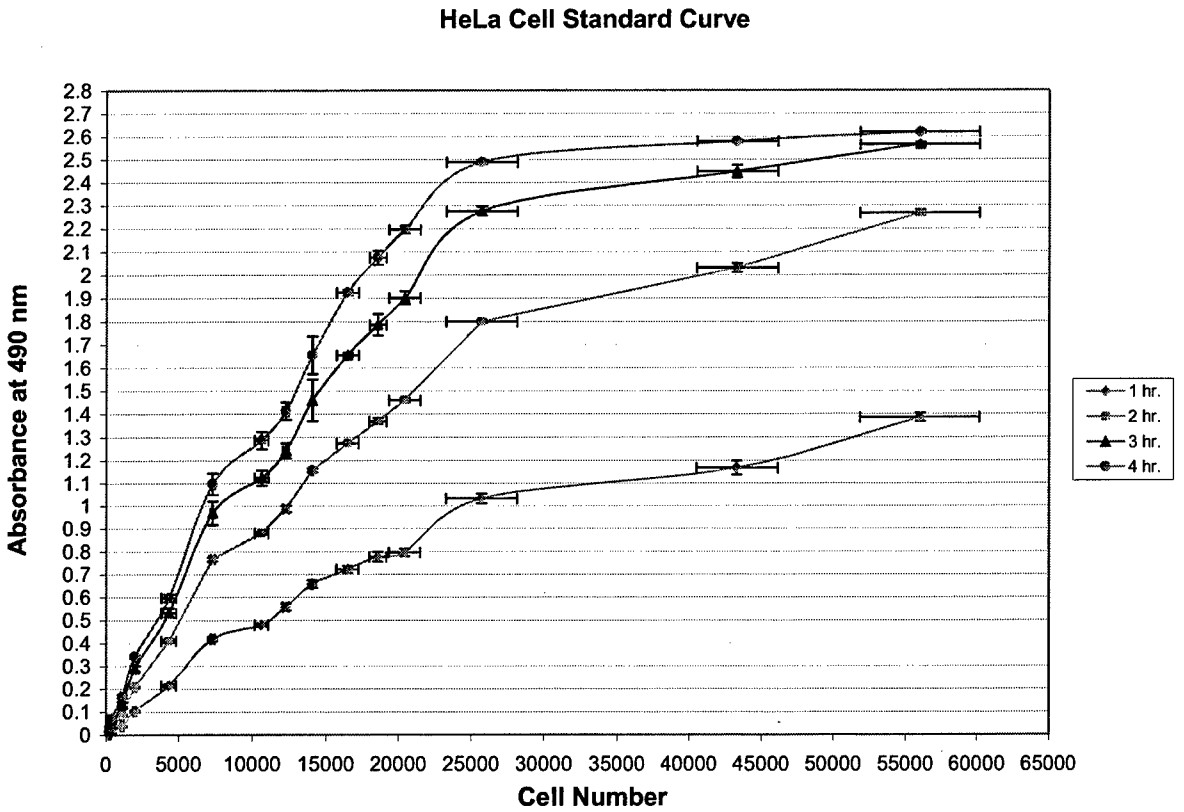

Figure 4. HeLa Cell Standard Curve. Mean of quadruplicate absorbance values at 490 nm ± SEM are shown at 1, 2, 3, and 4 hr after MTS addition.

3.2 Effect of MTS on Cell Number.

Cells were exposed to MTS for 25 hr. The results are shown in Figure 5. A 6 (cell number plated) x 2 (MTS absence or presence) Analysis of Variance (ANOVA) was performed. There was a significant interaction $F(5, 18) = 60.35$, $p<0.0001$, indicating that the main effect of MTS is not consistent over all levels of cell number plated. Therefore the simple effect of MTS absence or presence was tested at each level of cell number plated. A Bonferroni post hoc analysis showed that for cell numbers of 4000 or less, there was no significant effect of MTS on the dependent variable of final cell number ($p>0.05$). For cell numbers plated that were 8000 or more, there was a significant effect of MTS on final cell number ($p<0.001$).

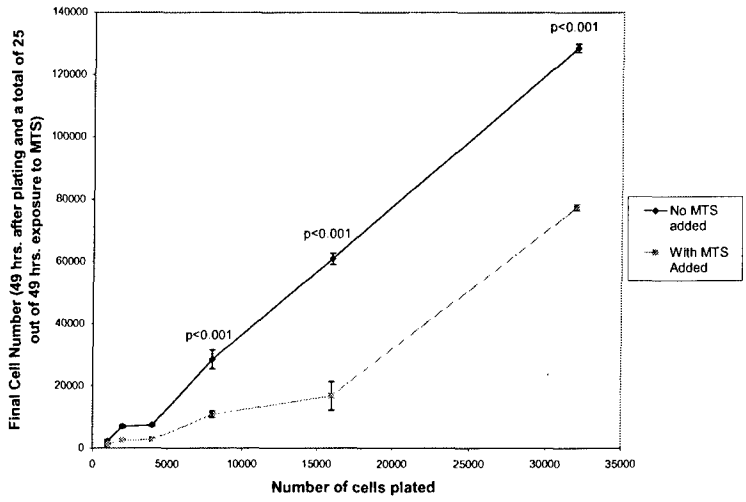

Figure 5. Exposure of HeLa Cells to the MTS Reagent for 25 hr. Average cell number is shown ± SEM. A two-way ANOVA was performed, and a significant interaction was found for Number of cells plated and MTS presence/absence $F(5,18) = 60.35$, $p<0.0001$. Therefore, Bonferroni post hoc analysis was completed for MTS presence or absence at different levels of number of cells plated. When the number of cells plated was 4000 or less, MTS had no significant effect on final cell number ($p>0.05$). For number of cells plated 8000 or more, MTS had a significant effect on final cell number ($p<0.001$).

3.3 Background Noise.

Figure 6 shows that when media with no phenol red is used instead of media with phenol red, the background absorbance level is significantly decreased.

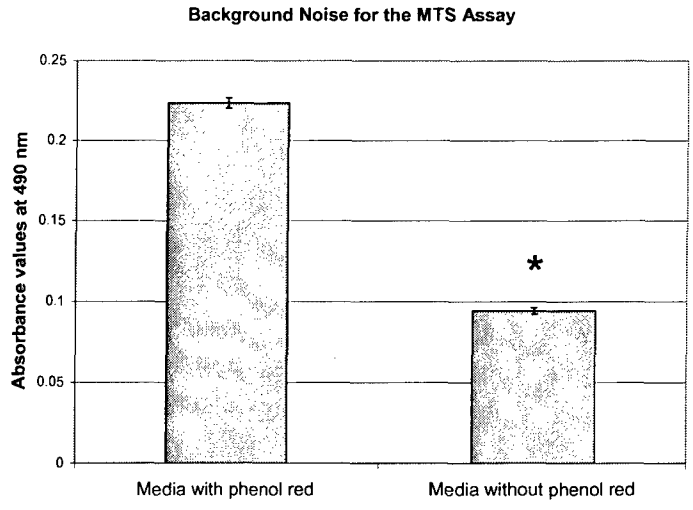

Figure 6. Media with No Phenol Red Reduces Background Signal *$p<0.001$. The mean of quadruplicate absorbance values ± SEM are shown.

4. DISCUSSION

The manufacturer of MTS recommends that the plate be read 1-4 hr after the addition of the reagent. The experiment completed here tested the possibility of completing experiments beyond that time point. Because the MTS reagent decreases cell number 25 hr after it was added for cell numbers plated 8000 cells/well or greater, reagent should not be added for this length of time under these cell population conditions.

The standard curves showed a linear range within a certain number of cells. In this assay, the background signal when media has no phenol red is approximately 0.05-0.09 absorbance units after 1 hr. This is the level of background, and we therefore concluded that the signal for the cells should start at least 0.1 absorbance units. When listing the recommended ranges below, the lower cutoff was the number of cells that gave at least 0.1 absorbance units at 1 hr after MTS addition and the upper range was approximated by determining when the graph departed from linearity and became saturated. These values are listed in the Table.

Table. Recommended Range of Cells for the MTS Assay for 4 cell lines

Cell line	Recommended number of cells
SK-N-SH	1800-7000
KYSE-30	2000-12,540
MCF-7	1000-10,000
HeLa	1000-25,700

The numbers above represent the minimum and maximum number of cells/well that should be present on the day of MTS addition and measurement. For example, if 7000 SK-N-SH cells were to be plated on day 1, by day 2 the cells would grow and be outside of the linear range of the assay. Thus, growth rate must be taken into account for each cell line.

The experiment of the effect of MTS on HeLa cell number showed an interaction of the two independent variables of cell number plated and MTS presence/absence. When a post hoc analysis was completed it revealed a shift from a non-significant effect (<4000 cells plated) to a significant effect (>8000 cells plated). It should be noted that if the dependent variable final cell number is examined, the values at >8000 cells/well plated are outside of the linear range of the MTS assay standard curve. The data points below 4000 cells/well plated are all within linear range according to the MTS assay standard curve. In the standard curve experiment for HeLa cells, the recommended linear range is 1000-25,700 cells. After 25,700 cells, a leveling off can be seen on the curve. This could be due to saturation and/or decreased cell growth rate due to media depletion. This experiment shows that the effect of a toxicant can differ depending on the cell number plated and emphasizes the importance of consistency across experiments in initial cell number plated. The standard curve experiments point out the importance of knowing whether or not the experiment is carried out in the linear range.

With respect to background noise for the assays, we determined that media without phenol red gave a significantly reduced background compared to media with phenol red, and we therefore recommended that this method be used with the assay.

LITERATURE CITED

1. Mosmann T. Rapid colorimetric assay for cellular growth and survival: Application to Proliferation and Cytotoxicity assays. *J. Immunol. Methods* **1983,** *65*, pp 55-63.

2. Slater T.F.; Sawyer B.; Strauli, U. Studies on Succinate-tetrazolium Reductase Systems. III. Points of Coupling of Four Tetrazolium Salts. *Biochim. Biophys. Acta* **1963**, 77, pp 383-393.

3. Berridge, M.V.; Tan, A.S. Characterization of Cellular Reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT): Subcellular Localization, Substrate Dependence, and Involvement of Mitochondria Electron Transport in MTT Reduction. *Arch Biochem Biophys.* **1993**, *303 (2)*, pp 474-482.

4. Promega Corporation. (1999). CellTiter 96® Aqueous One Solution Cell Proliferation Assay. *Technical Bulletin* No. 245.

5. Berg K.; Zhai L.; Chen M.; Kharazmi A.; Owen, T.C. The use of a water-soluble formazan complex to quantitate the cell number and mitochondrial function of Leishmania major promastigotes. *Parasitol Res.* **1994**, *80 (3)*, pp 235-239.

6. Shimada, Y.; Imamura, M.; Wagata, T.; Yamaguchi N.; Tobe, T. Characterization of 21 newly established esophageal cancer cell lines. *Cancer* **1992**, *69 (2)*, pp 277-284.